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Histopathological Study of Time Course Changes in Obstructed Lymphatic Vessels in Rats

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Abstract

Background: Lymphedema is a progressive accumulation of protein-rich fluid in the interstitial space of the skin, resulting from an anatomic or functional obstruction in the lymphatic system. Currently effective therapies for this disease are little known. We have been using surgical treatment, lymphaticovenular anastomoses, for patients and experienced some cases that improved dramatically and did not require the use of stockings and massages for more than ten years after surgery.

Methods: We investigated transitional morphological changes in lymphatic obstructed models by electron microscopy with focus on lymphatic smooth muscle cells after experimentally lymphatic vessel occlusion.

Results: We observed interesting dramatic alterations in obstructed lymphatic smooth muscle cells at different times.

Conclusion: These results suggest possibilities for the diversity of lymphatic smooth muscle cells.

Introduction

Lymph edema is a chronic debilitating disease that results from impairment of the lymphatic system. There are various causes of lymphatic system impairment, including congenital lymphatic anomaly, infection, lymphatic injury, and surgery. We have been using surgical treatment, lymphaticovenular anastomoses, for patients with severe edema who show little improvement with conservative treatment using an elastic stocking and/or elastic bandage [1-3]. We experienced some cases that improved dramatically and did not require the use of stockings and massages for more than ten years after surgery (data not shown). It is not clear whether it depends on the lymphatic functional regeneration by our treatments, the lymphatic compensation mechanism by residual lymphatic system or others.

In this study, we investigated transitional morphological changes in lymphatic obstructed models by electron microscopy with focus on Lymphatic Smooth Muscle Cells (LSMCs) after experimentally lymphatic vessel occlusion. As for the current studies of lymphatic system, cancer research-related lymph angiogenesis, the metastatic spread, drug discovery and so on are done flourishingly in various fields. In the 1990s, the discovery of the Vascular Endothelial Growth Factor (VEGF) family of proteins played a crucial role in lymphatic research. The receptor VEGF-3 and its ligands, VEGF-C and VEGF-D, were soon identified and they promote lymph angiogenesis by activating the VEGF receptor-3, which is expressed on lymphatic endothelial cells [4-9]. The current focus of lymphatic research is lymphatic endothelial cells. We targeted LSMCs, which are novel approach. This is the first study to demonstrate transitional histopathological changes in LSMCs, and we hypothesized that lymphatic function which had been extinct once would regenerate.

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Materials and Methods

Sixteen male Wistar rats (Charles River Japan, Yokohama) weighing 250-300 g were used in this study. These experimental protocols were approved by the Ethics Committee for Animal Experiments in University of Tokyo Medical School. All operations were performed under general anesthesia with pentobarbital sodium administered intraperitoneally at 50 mg/kg body weight. The surgical procedure was performed using an operating microscope. The experiment was divided into two sections: normal morphological study and Histopathological study.

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Morphological study and harvest of normal lymphatic (n=3)

First, a rectangular-shaped incision was made in the tail of each rat at the dorsal midline side, and sub connective tissue was separated to free the skin flap and then the lateral neurovascular bundle was exposed [Figure 1a and 1b]. Some lymphatic vessels accompanying with the bundle were identified and then the each of the lymphatic was carefully isolated (Figure 1c) and ligated with 10-0 nylon. To minimize the influence of surgical trauma on the vasospasm model, we removed very little perivascular and adventitial tissue. Normal lymphatic vessels were harvested.

Histopathological study (n=15)

The skin flap could be opened repeatedly. Samples of approximately 2 - 3 mm in length were dissected. Obstructed lymphatic's were harvested at different time points (6 hours, 1 day, 4 days, 7 days) and the skin flap were simply closed with an 5-0 suture.

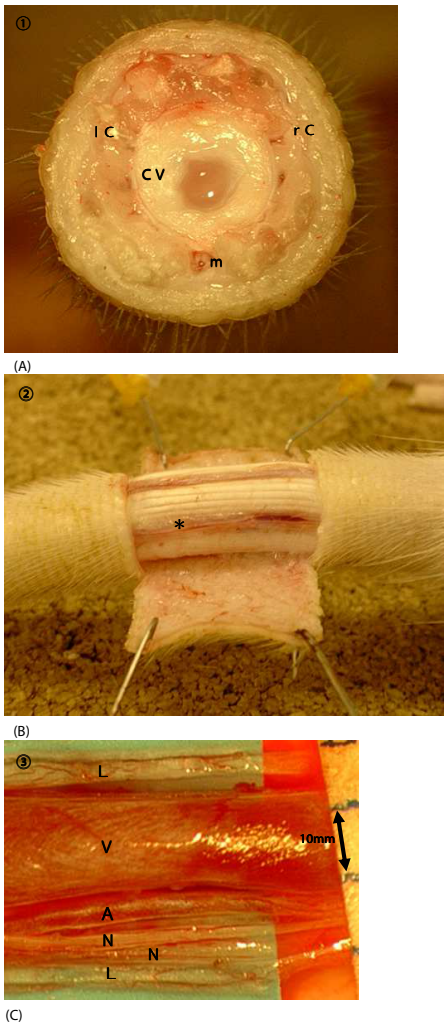

Figure 1: (a) Transverse section of the rat tail. rC, right superficial lateral neurovascular bundle; lC, light superficial lateral neurovascular bundle; m, middle caudal neurovascular bundle; CV, caudal vertebra. (b) Two symmetrical skin flap windows were raised in the rat tail at the middle dorsal side. Single asterisks showed the right superficial lateral caudal neurovascular bundles. (c) Close up view of the neurovascular bundle as seen through the operating microscope (original magnification × 20). L, lymphatic vein; A, artery; V, vein; N, nerve.

Transmission Electron Microscopy (TEM)

Samples were fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4 γ and then post fixed with 1% osmium tetroxide in cacodylate buffer (pH 7.4) for 1 h at 4 α. After staining with 1% aqueous uranyl acetate solution for 10 min at room temperature, the samples were dehydrated in a graded series of ethanol solutions. For transmission electron microscopy, the blocks were embedded in Epon after dehydration. Ultrathin sections were cut at 5.0 nm with a diamond knife, stained with urany acetate and lead citrate, and observed under a transmission electron microscope (H-7000; Hitachi, Tokyo, Japan) at an acceleration voltage of 75 kV.

Results

Lymphatic architecture in the rat tail

Anatomic dissection studies revealed that the lymphatic vessels have an average diameter of 0.3 mm (range, 0.2-0.5 mm) and are transparent and flexible. We observed lymphatic vessels in rat tails by transmission electron microscopy with focus on alterations of smooth muscle cells (Figure 2).

Normal lymphatic findings [10]

The lymphatic system can be divided into several parts according to histological structure of the vessel wall. From the distal site, the system consists of initial lymph vessels, lymph capillaries, precollectors, collectors and lymph trunks, which join the venous system. The lymphatic vessels we harvested were collectors, the walls of which consist of three layers: a tunica interna lined with an endothelial layer, a tunica media composed of smooth muscle cells and a tunica externa (Figure 2c). However, these layers are not clearly distinguishable because lymphatics do not possess any elastic lamina observed in collector veins and arteries (Figure 2a and 2b). The number of smooth muscle cells in lymphatic vessels is less than that in arteries and veins (Figure 2c).

Normal lymphatic smooth muscle cell findings by TEM

Smooth Muscle Cells (SMCs), surrounding by fascicles of collagen were seen in the media. They were invested with basal lamina and were long fusiform cells with typical myofilaments, dense bodies, and few organelles, such as Golgi apparatus, rough endoplasmic reticulum and mitochondria (Figure 2c).

Histopathological findings of time course changes in obstructed lymphatic smooth muscle cells

We observed interesting dramatic alterations in obstructed lymphatic smooth muscle cells at different times. At 6 hours, the lymphatic vessel was a little dilated (Figure 3a) and LSMCs showed circumferential elongation, loss of surface corrugation, a few surface vesicles and some mitochondria (Figure 3b). At 24 hours, ultrastructually LSMCs had lost their normal fusiform shape and were separated by an increased amount of irregularly disposed extracellular collagen fibers. Cell membranes appeared rolling with membrane-bound vesicles and a large number of mitochondria (Figure 4a). While the proportion of myofibrils was reduced, it occupied the majority of the cell. LSMCs also showed morphological expressions of phagocytosis of collagen, elastic fibers and themselves and in part of LSMCs apoptosis bodies were detected (Figure 4b). At 4 days, the lymphatic vessels were more dilated and lost transparency and flexibility (Figure 5a). LSMCs appeared conspicuously-enlarged distal

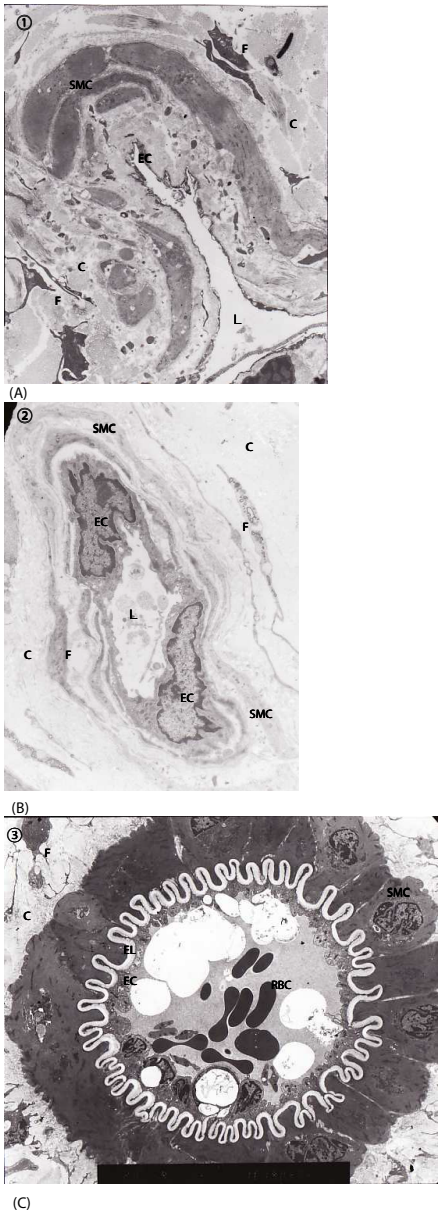

Figure 2: Transmission electron micrograph of a lymphatic vessel, a vein and an artery in a normal rat tail. (a) Lymphatic vessel. SMC, LSMC; L, lumen; EC, endothelial cell; C, collagen; F, fibroblast. Original magnification × 4,000. (b) Vein. SMC, vascular smooth muscle cell; L, lumen; EC, endothelial cell; C, collagen; F, fibroblast. Original magnification × 3,000. (c) Artery. SMC, vascular smooth muscle cell; EL, elastic lamina; L, lumen; RBC, red blood cell; EC, endothelial cell; C, collagen; F, fibroblast. Original magnification × 4,000.

to the ligated point. Ultra structurally the size of smooth muscle cells was smaller and the percentage of myofibrils was further reduced with irregular cell membrane, considerable surface vesicles and well-developed endoplastic reticulum, but form of grating of myofibrils is still preserved. Both endothelial cells and smooth muscle cells showed evidence of endocytotic activity (Figure 5b and 5c). At 7 days, severely damaged LSMCs with almost complete loss of the cellular lattice-like network were observed. It is impossible to find the profile of the cells. Fibroblasts collagen fibrils increased dramatically and filled the lymphatic lumen (Figure 6).

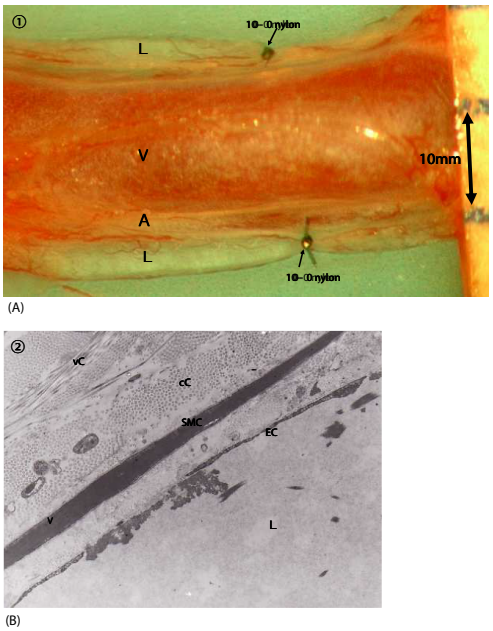

Figure 3: Gross photograph (a) and TEM (b) of an obstructed lymphatic vessel in 6 hours. (a) 10-0 nylon (arrow), L, LSMC; A, artery; V, vein; N, nerve. (b) LSMCs appeared stretched. SMC, smooth muscle cell; L, lumen; EC, endothelial cell; vC, vertical collagen; cC, cross collagen; v, vesicles. Original magnification × 18,000.

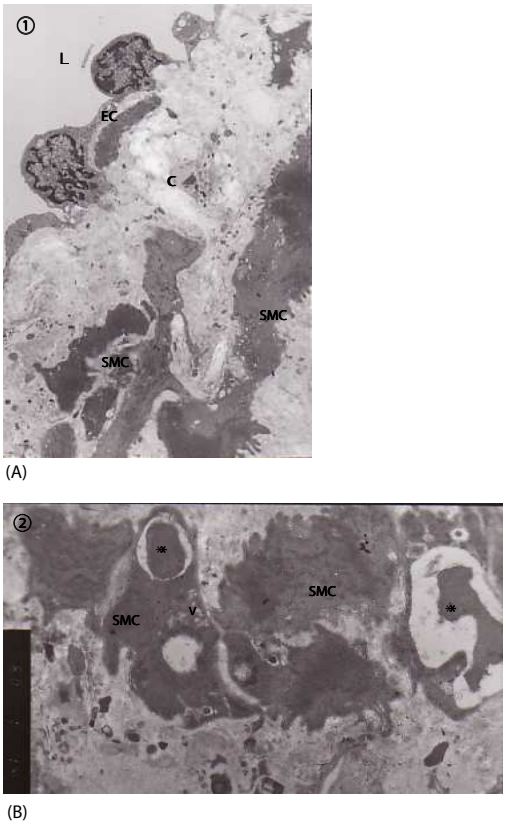

Figure 4: TEM of an obstructed lymphatic vessel in 24 hours. (a), (b) LSMCs showed morphological expression of phagocytosis of collagen, elastic fibers and themselves (single asterisks). SMC, LSMC; L, lumen; EC, endothelial cell; C, collagen; v, vesicles; asterisk, apoptotic body. Original magnification × 8,000.

Discussion

Lymph flow in collectors depends predominantly on lymphatic contraction [11-12]. Intrinsic generation of action potentials within the smooth muscle induces spontaneous contraction [13-17]. The rate of lymph transport can be significantly affected by humoral and physical factors that influence the rhythm and amplitude of spontaneous constrictions [18]. Activation of β-adrenoreceptors has been shown to decrease the frequency and force of spontaneous constrictions of bovine mesenteric lymphatic vessels [19]. Oxygen free radicals [20] and endothelin-derived nitric oxide [21] reduce the efficacy of action potential generation of lymphatic smooth muscle pacemaker potentials and, hence, lymphatic phasic constrictions. Lymphatic flow and lymphatic contractility increase in response to tissue edema (edema safety factor), exercise, hydrostatic pressure (standing position), mechanical stimulation (massage, pneumatic compression), and local hyperthermia [22].

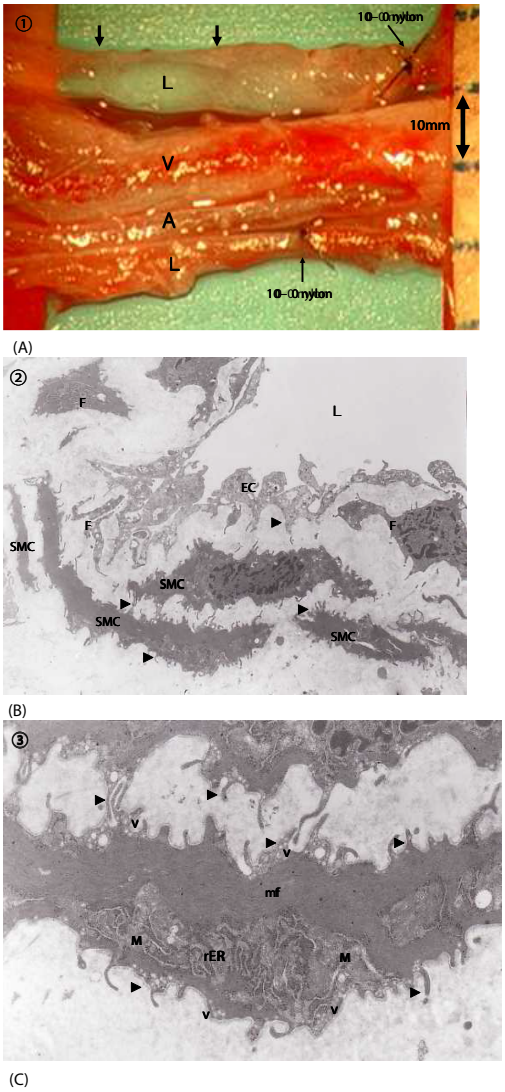

Figure 5: Gross photograph (a) and TEM (b), (c) of an obstructed lymphatic vessel in 4 days. (a) LSMCs appeared conspicuously-enlarged distal to the ligated point. L, LSMC; A, artery; V, vein; arrow, valve. (b) Both endothelial cells and smooth muscle cells showed evidence of endocytotic activity (arrowheads). SM, LSMC; L, lumen; EC, endotherial cell; F, fibroblast; C, collagen. Original magnification × 3,000. (c) m, myofilament component; rER, rough endoplasmic reticulum; M, mitochondria; v, vesicles; F, fibroblast. Original magnification × 15,000.

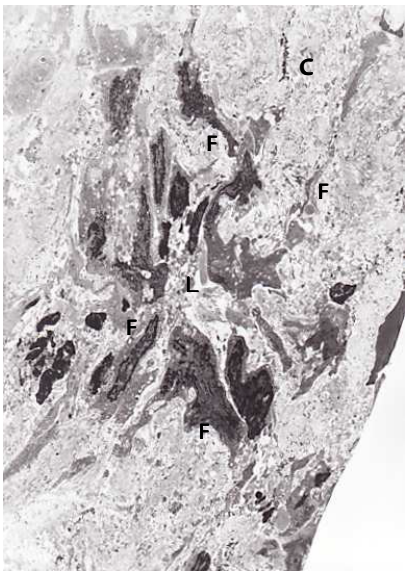

Figure 6: TEM of an obstructed lymphatic vessel in 7 days. Fibroblasts collagen fibrils increased dramatically and filled the lymphatic lumen. L, lumen; F, fibroblast; C, collagen. Original magnification × 5,000.

In this study, we made morphological observations of obstructed lymphatics with a transmission electron microscopy. After lymphatic obstruction, LSMCs dramatically changed in phenotype, from one with abundant myofilaments to a phenotype with loss of myofilaments as well as increased number of secretory organelles (such as the rough endoplasmic reticulum and Golgi) and formation of apoptotic bodies. These findings strongly suggested a sign of remodeling of the lymphatic vessel wall and resemble the pathogenesis of atherosclerosis and restenosis after angioplasty, the diversity of smooth muscle cells [23], [24]. Unlike mature cardiac and skeletal muscle cells, which are terminally differentiated and incapable of further cell cycle activity, mature Vascular Smooth Muscle Cells (VSMCs) retain the ability to convert reversibly in response to appropriate environmental stimuli, such as stretching and shear stress. Depending on the environment, VSMCs can switch their phenotype between a motile, proliferative, "synthetic" state and a mature "contractile" state. This plasticity in VSMCs is critically involved in the development of atherosclerosis and vascular stenosis [23-27]. We speculated that a similar pathologic condition could occur in the lymphatic system, that is, modulate from a contractile phenotype to synthetic phenotype, resulting in lymphatic sclerosis such as atherosclerosis (Figure 7). Additionally, lymphatic system has superior potential of regeneration and/or adaptive response to vascular system. We can reconstruct vascular flow, nerve and muscle function, and bony union following replantation surgery and microvascular free-flap operation, but not lymphatic flow. Although temporary, acute postoperative swelling of a replanted part is attributed to lymph edema, this condition resolves without microsurgical intervention. Spontaneous regeneration or reconnection of lymphatics [28] and/or dilatation of pre-existing small lymphatic vessels are thought to occur in such situations [29]. However, in the setting of chronic lymphedema, current evidence suggests that dysfunctional lymphatic vessels do not undergo any functional improvements over time. Therefore, the functional improvement of lymphatic vessels themselves could be an important therapeutic strategy for the

prevention and treatment of lymphedema. The plasticity of LSMCs might be a new target for the prevention and treatment of lymph edema (Figure 7). Further analyses are necessary to characterize the lymphatic phenotype to determine the reversibility of lymphatic remodeling.

Currently, it is difficult to predict before surgery which patients will be afflicted. Those who came back alive from carcinoma are afraid that they will be distressed by lymph edema in future and those who do not yet develop lymph edema worry that their condition will be worse.

Further studies on lymphatic change in lymph edema are required for proving the above hypothesis. Thus, the present study represents the first step in exploring the potential of the lymphatic system, especially lymphatic smooth muscle cells.

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